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Abstract: MYC rearrangement can be detected in a subgroup of diffuse large B-cell lymphoma characterized by unfavorable prognosis. In contrast to Burkitt lymphoma, the correlation between MYC rearrangement and MYC protein expression in diffuse large B-cell lymphoma is less clear, as approximately one-third of rearranged cases show negative or low expression by immunohistochemistry. To better understand whether specific characteristics of the MYC rearrangement may influence its protein expression, we investigated 43 de novo diffuse large B-cell lymphoma positive for 8q24 rearrangement by FISH, using 14 Burkitt lymphoma for comparison. Different cell populations (clones), breakpoints (classical vs non-classical FISH patterns), partner genes (IGH vs non-IGH) and immunostaining were detected and analyzed using computerized image systems. In a subgroup of diffuse large B-cell lymphoma, we observed different clones within the same tumor distinguishing the founder clone with MYC rearrangement alone from other subclones, carrying MYC rearrangement coupled with loss/extra copies of derivatives/normal alleles. This picture, which we defined MYC genetic heteroclonality, was found in 42% of cases and correlated to negative MYC expression (P=0.026). Non-classical FISH breakpoints were detected in 16% of diffuse large B-cell lymphoma without affecting expression (P=0.040). Non-IGH gene was the preferential partner of rearrangement in those diffuse large B-cell lymphoma showing MYC heteroclonality (P=0.016) and/or non-classical FISH breakpoints (P=0.058). MYC heteroclonality was not observed in Burkitt lymphoma and all cases had positive MYC expression. Non-classical FISH MYC breakpoint and non-IGH partner were found in 29 and 20% of Burkitt lymphoma, respectively. In conclusion, MYC genetic heteroclonality is a frequent event in diffuse large B-cell lymphoma and may have a relevant role in modulating MYC expression.Modern Pathology advance online publication, 29 April 2016; doi:10.1038/modpathol.2016.71.

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Definition of MYC genetic heteroclonality in diffuse large B-cell lymphoma with 8q24 rearrangement and its impact on protein expression

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MYC rearrangement can be detected in a subgroup of diffuse large B-cell lymphoma characterized by unfavorable prognosis. In contrast to Burkitt lymphoma, the correlation between _MYC_ rearrangement and MYC protein expression in diffuse large B-cell lymphoma is less clear, as approximately one-third of rearranged cases show negative or low expression by immunohistochemistry. To better understand whether specific characteristics of the _MYC_ rearrangement may influence its protein expression, we investigated 43 _de novo_ diffuse large B-cell lymphoma positive for 8q24 rearrangement by FISH, using 14 Burkitt lymphoma for comparison. Different cell populations (clones), breakpoints (_classical vs non-classical_ FISH patterns), partner genes (_IGH vs_ non-_IGH_) and immunostaining were detected and analyzed using computerized image systems. In a subgroup of diffuse large B-cell lymphoma, we observed different clones within the same tumor distinguishing the _founder_ clone with _MYC_ rearrangement alone from other subclones, carrying _MYC_ rearrangement coupled with loss/extra copies of derivatives/normal alleles. This picture, which we defined _MYC genetic heteroclonality_, was found in 42% of cases and correlated to negative MYC expression ($P = 0.026$). _Non-classical_ FISH breakpoints were detected in 16% of diffuse large B-cell lymphoma without affecting expression ($P = 0.040$). Non-_IGH_ gene was the preferential partner of rearrangement in those diffuse large B-cell lymphoma showing _MYC heteroclonality_ ($P = 0.016$) and/or _non-classical_ FISH breakpoints ($P = 0.058$). _MYC heteroclonality_ was not observed in Burkitt lymphoma and all cases had positive MYC expression. _Non-classical_ FISH _MYC_ breakpoint and _non-IGH_ partner were found in 29 and 20% of Burkitt lymphoma, respectively. In conclusion, _MYC genetic heteroclonality_ is a frequent event in diffuse large B-cell lymphoma and may have a relevant role in modulating MYC expression.

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Diffuse large B-cell lymphoma is the most common mature aggressive B-cell lymphoma. Despite recent therapeutic achievements, approximately 40% of diffuse large B-cell lymphoma cannot be cured, which indicates the need for further studies focusing on the characterization of the putative genes involved in diffuse large B-cell lymphoma pathogenesis. In this context, *MYC* has a relevant role, and the presence of *MYC* rearrangement, described in approximately 5 to 15% of diffuse large B-cell lymphoma cases, correlates with worse prognosis and poor response to R-CHOP treatment.[1–5]

A novel commercial monoclonal antibody has improved the detection of MYC protein expression by immunohistochemistry in formalin-fixed paraffin-embedded tissues. Using this antibody, Burkitt

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lymphoma with *MYC* rearrangement were found to show intense positive staining for MYC protein in the vast majority of the cells, supporting the existence of a strong correlation between gene and protein status.[6–7] In contrast, in *MYC* rearranged diffuse large B-cell lymphoma, the association between rearrangement and protein expression is less clear, as approximately one-third of cases with this rearrangement show protein staining in $<40\%$ of the tumor cells.[5,8–12]

Conventional and molecular cytogenetic studies of diffuse large B-cell lymphoma have described cases with *MYC* rearrangement that are characterized by complex karyotypes, revealing the presence of different populations of cells with loss/extra copies of derivatives of the *MYC* rearrangement and/or extra copies of chromosome 8.[13–18] The occurrence of these alterations and their contribution to protein expression remains unknown.

To further complicate this picture, B-cell lymphomas with *MYC* rearrangement show a great variability in both the location of the breakpoint in the 8q24 region (involving or not the *MYC* coding region) and the partner gene implicated in the translocation. Non-*IGH* loci have been reported as the most common partners in diffuse large B-cell lymphoma.[4,16,19] In addition, when non-*IG* loci are involved, breakpoints are usually located outside of the *MYC* coding region.[15,20–25] The effects of the different *MYC* breakpoints and/or rearrangement partners on MYC expression are not well understood, but recent studies revealed that the *IG* partner (mainly represented by the *IGH* gene) is a negative predictor of survival in diffuse large B-cell lymphoma.[19,26] Finally, concomitant *BCL2* and/or *BCL6* rearrangements in so-called double/triple-hit lymphomas have been reported in the context of the diffuse large B-cell lymphoma complex karyotypes.[1,4,16]

To better understand whether specific characteristics of *MYC* genetic alterations may influence its protein expression, we performed a detailed analysis of *MYC* rearrangement using new computerized imaging systems in a series of *de novo* diffuse large B-cell lymphoma. We described and quantified different populations of cells with *MYC* rearrangement and loss/extra copies of the derivatives of the translocation and/or of the normal allele. Different types of *MYC* breakpoints and their correlation to *IGH* as a translocation partner were also investigated. MYC protein staining was evaluated and compared with all findings.

Materials and methods

Case Selection

A total of 43 *de novo* diffuse large B-cell lymphoma cases characterized by *MYC* rearrangement in 8q24 were retrieved from a series of B-cell lymphomas investigated for diagnostic purposes during the last 15 years at the Institute of Pathology, Locarno (Switzerland), Laboratories of Pathology from the Hospital Clínic of Barcelona and the Hospital del Mar, Barcelona (Spain), and Institute of Pathology at the University Hospital, Basel (Switzerland). All tumors were diagnosed according to World Health Organization (WHO).[27] Formalin-fixed paraffin-embedded tissue was available for all cases. Clinical information and follow-up data were obtained for 25 diffuse large B-cell lymphoma patients. To compare our results with another group of mature aggressive B-cell lymphomas with *MYC* rearrangement, 14 Burkitt lymphoma cases were included in this study.

Immunohistochemistry

Immunohistochemical studies were performed with a panel of monoclonal and polyclonal antibodies using a peroxidase-labeled detection system, standard antigen retrieval protocols and an automated immunostainer (Roche Ventana Medical Systems, Baar, Switzerland and Dako Autostainer, Dako, Copenhagen, Denmark). The panel of antibodies used included CD20, CD79a, CD10, BCL6, MUM1, BCL2 and Ki-67 (Supplementary Table 1). *In situ* hybridization was performed to analyze the presence of Epstein–Barr virus (EBV). For all of these markers, a cutoff of 25% stained cells was used to interpret the results as positive, similar to previously reported studies, with the exception of BCL2, for which a cutoff of 50% was used.[8] Diffuse large B-cell lymphoma were subclassified into GCB and non-GCB according to the Hans' algorithm.[28]

MYC protein expression was assessed using the ready-to-use monoclonal antibody (clone Y69) following the manufacturer's protocol (Roche Ventana Medical Systems) and evaluated with a computerized system. Digital images were captured and analyzed with an Aperio Image Scope (Aperio Technologies, Vista, CA, USA). Areas of artifacts, necrosis and other poor quality regions were excluded from analysis. Expression in $>40\%$ of tumor cells was used as a cutoff to classify a case as MYC positive, as reported in the literature.[7–9]

Fluorescence *In Situ* Hybridization

FISH was performed on 3- to 4- μm-thick formalin-fixed paraffin-embedded tissues sections following current recommendations, as previously described.[29,30] The commercially available *MYC* split signal probe from Dako was applied to all cases (*MYC* FISH DNA Probe, Split Signal, Dako). This probe was designed to hybridize upstream and downstream of the *MYC* breakpoint cluster region with a green probe (that binds to a 652-kb segment centromeric to the *MYC* breakpoint cluster region on chromosome 8q24) and a red probe (that binds to a 418-kb segment telomeric to

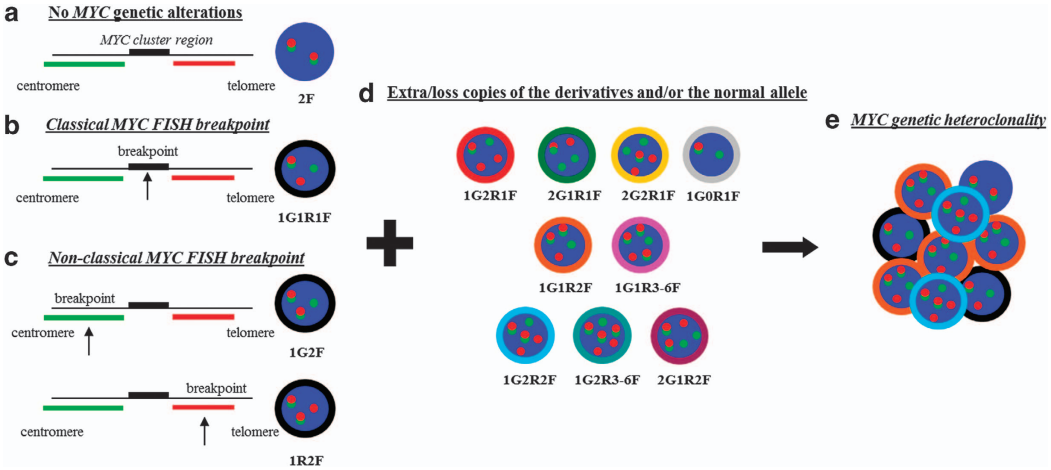

Figure 1 Schematic representation of FISH signal patterns using *MYC* split signal probe in a *MYC* normal cell (**a**), in a *MYC* rearranged cell with classical (**b**) or non-classical FISH *MYC* breakpoints (**c**) (probe design and breakpoints definition should be intended using the Dako Split Signal probe). Complex FISH patterns showing additional *MYC* alterations (**d**). Population carrying *MYC* rearrangement alone (founder clone, **b** or **c**) and derived subclones with *MYC* complex pattern (**d**) resulting in *MYC genetic heteroclonality* (**e**).

the *MYC* breakpoint cluster region), respectively, with a gap of approximately 94 kb. Colocalization of the probes results in a red/green signal (fusion, 1F) indicating normal *MYC* allele, thus a normal cell display a 2 F FISH pattern (Figure 1a). Translocation events in the *MYC* breakpoint cluster region will split one fusion signal in separate green (1G) and red (1R) signals, thus resulting in a cell with 1G1R1F FISH pattern (Figure 1b). As a result of this specific probe design (http://www.dako.com), breaks occurring in the sequences centromeric or telomeric to the *MYC* breakpoint cluster region result in 1G2F or 1R2F FISH pattern, respectively (Figure 1c). Cells carrying one of these different FISH signals patterns were considered positive for the presence of *MYC* rearrangement. For simplification, on the basis of FISH pattern, cells with 1G1R1F signals were classified as carrying a *classical FISH MYC breakpoint*, whereas those with 1G2F or 1R2F signals were grouped as having a *non-classical FISH MYC breakpoint*.

In selected cases, *MYC* was also evaluated by means of another break-apart probe (Vysis *MYC* Break Apart FISH Probe Kit, Abbott Molecular, IL, USA), which was similarly designed with a dual color strategy, but covering a larger region at 8q24 with a gap of approximately 1.6 Mb. To identify the *IGH* locus as a potential partner for *MYC* rearrangement, an *IGH/MYC* dual fusion probe (Vysis LSI *IGH/MYC*, CEP 8, Tri-color Dual Fusion Translocation Probe, Abbott Molecular) was used in cases with available material. FISH for *BCL2* and *BCL6* using split signals probes were performed for most diffuse large B-cell lymphoma cases (*BCL2* and *BCL6* FISH DNA Probe, Split Signal, Dako).

Only cases with at least 90% hybridization efficiency were considered. FISH signal patterns were interpreted following the criteria of Ventura *et al*.[31] Ten tonsil sections were used as negative controls. The cutoff used to establish the presence of rearrangements with the split signal and dual fusion probes was 5%.[29,31]

All FISH experiments were evaluated with a conventional fluorescence microscope by two expert operators (AV and VM). In addition, all hybridizations performed with the *MYC* Dako split signal probe were analyzed with the Bioview image-based quantification system (BioviewDuet^{TM}, NesZiona, Israel). A minimum of 100 cells with clear non-overlapping nuclei were selected and automatically scored for each case. Tissue matching was performed for the H&E, FISH and immunohistochemistry slides to ensure that the same tumor areas were evaluated.

Statistical Analysis

Categorical data were compared using Fisher's exact test with two-sided *P*-values, and for ordinal data, nonparametric tests were used. Statistical analysis was performed using the SPSS software package version 21.0 (SPSS, Chicago, IL, USA).

Results

Clinical and Pathological Characteristics of Diffuse Large B-Cell Lymphoma

Forty-three patients, including 31 males and 12 females, with a mean age of 68 years (range of 38–93 years) were examined in this study. One (2%) patient had HIV infection, 22/43 (51%) had extranodal presentation and 9/25 (36%) cases died of disease. All cases showed CD20 and/or CD79a expression. CD10 was positive in 32/42 (76%), BCL6 in 36/40 (90%) and MUM1 in 12/34 (35%). BCL2 expression was detected in 34/41 (83%) cases. Thirty-two of 40 (80%) cases had a GCB immunophenotype. A high proliferation index (Ki-67 > 90%)

Table 1 Detailed FISH patterns using *MYC* split signal probe and MYC expression in diffuse large B-cell lymphoma with *MYC genetic heteroclonality*

Case	*MYC rearrangement*		*MYC rearrangement +extra normal allele*		*MYC rearrangement +extra/loss derivatives*		*MYC rearrangement +extra normal allele +extra derivatives*		*No MYC rearrangement*		*MYC IHC*
	% Of cells	*Pattern*	*% Of cells*	*Pattern*	*% Of cells*	*Pattern*	*% Of cells*	*Pattern*	*% Of cells*	*Pattern*	*% Of cells*
1	27%	1G1R1F	36%	1G1R2F	—	—	—	—	37%	2F	97%
2	32%	1G1R1F	55%	1G1R2F	—	—	—	—	13%	2F	NE
3	46%	1G1R1F	45%	1G1R2F	—	—	—	—	9%	2F	35%
4	10%	1G1R1F	90%	1G1R3F	—	—	—	—	—	—	26%
5	18%	1G1R1F	10%	1G1R2F	50%	2G2R1F	—	—	22%	2F	8%
6	30%	1G1R1F	23%	1G1R2F	14%	1G2R1F	16%	1G2R2F	17%	2F	92%
7	22%	1G1R1F	22%	1G1R2F	—	—	46%	1G2R2F	10%	2F	86%
8	9%	1G1R1F	28%	1G1R2F	—	—	27%	2G1R2F	36%	2F	35%
9	19%	1G1R1F	22%	1G1R2F	—	—	53%	2G1R2F	6%	2F	3%
10	7%	1G1R1F	5%	1G1R2-4F	—	—	57%	1G2-3R2-4F	31%	2F	13%
11	5%	1G1R1F	49%	1G1R3-6F	—	—	17%	1G2R3-6F	11+18%	2F+3-6F	92%
12	20%	1G1R1F	—	—	22%	2G1R1F	35%	2G1R2F	23%	2F	8%
13	60%	1G1R1F	—	—	25%	2G1R1F	—	—	15%	2F	24%
14	20%	1G1R1F	—	—	80%	1G2R1F	—	—	0%	2F	98%
15	9%	1G1R1F	—	—	61%	1G0R1F	—	—	30%	2F	20%

Abbreviations: F, fusion signal (normal *MYC* allele); G, green signal (*MYC* centromeric derivative); IHC, immunohistochemistry; NE, not evaluable; R, red signal *(MYC* telomeric derivative).
Clones dimensions are intended as the percentage of tumor cells carrying the specific pattern or staining, both evaluated by computerized strategy. For simplification, only cases with *classical FISH MYC breakpoint* are listed (full raw data available in Supplementary Table 2).

Table 2 Clinical, pathological and genetic features in diffuse large B-cell lymphoma regarding *MYC genetic heteroclonality*

	MYC genetic (n = *43)*	
	*Heteroclonality (*n = *18)*	*No heteroclonality (*n = *25)*
Clinical		
> 60 years	15/18 (83%)	15/25 (60%)
Gender (M:F)	14:4	17:8
Dead of disease	4/11 (36%)	5/14 (26%)
Extranodal location	11/18 (61%)	11/25 (44%)
Immunohistochemical		
CD10+	15/18 (83%)	17/24 (71%)
BCL6+	17/18 (94%)	19/22 (86%)
MUM1+	3/15 (20%)	9/19 (47%)
GCB phenotype	15/17 (88%)	17/23 (74%)
BCL2+	16/18 (89%)	18/23 (78%)
MYC+*	8/17 (47%)	20/25 (80%)
Genetics		
IGH/MYC+**	6/14 (43%)	18/22 (82%)
Double/triple hit	12/15 (80%)	15/21 (71%)

*$P = 0.026$, **$P = 0.016$.

was observed in 25/41 (61%) cases. EBV infection was present in 1/24 (4%) case.

Twenty-eight of 42 (67%) cases were classified as positive for MYC expression, which was observed in a range of 42–98% tumor cells, with mean and median values of 77% and 86%, respectively. Fourteen cases (33%) were classified as negative for MYC expression, with a range of 3–35% stained cells and mean and median values of 18% and 17%, respectively.

Genetic Characteristics of Diffuse Large B-Cell Lymphoma

According to FISH patterns of *MYC* translocation, two subgroups of diffuse large B-cell lymphoma were identified (all raw data available in Supplementary Table 2). Twenty-five of 43 (58%) diffuse large B-cell lymphoma showed a single pattern of rearrangement characterized by one copy of a normal *MYC* allele and two derivatives (Figures 1b and 1c), which was observed in 40–98% of the tumor cells (mean: 71% and median: 73%). We termed this population of cells as *'MYC founder* clone' (ie, cells showing *MYC* rearrangement as the sole abnormality). In contrast, 18/43 (42%) cases had the *MYC founder* clone (5–60% of cells; mean: 22% and median: 20%) in combination with two or more clones harboring *MYC* rearrangement coupled with additional alterations (extra copies of the normal allele, extra copies of derivatives, extra copies of both, or deletion of one of the derivatives; Figure 1d). We called this complex distribution of *MYC* rearrangement *MYC genetic heteroclonality* (*MYC HC*) (Figure 1e). Table 1 shows the FISH patterns details of cases characterized by *MYC HC*. No clinical or pathological differences were observed in the diffuse large B-cell lymphoma according to these two subgroups (detailed in Table 2).

BCL2 and *BCL6* rearrangements were detected in 19/36 (53%) and in 13/34 (38%) cases, respectively. Overall, double hit characterized 19/33 (58%) cases (12 *MYC* and *BCL2*; 7 *MYC* and *BCL6*) and triple hit characterized 5/33 (15%) cases. The presence of

Table 3 Clinical, pathological and genetic features in diffuse large B-cell lymphoma regarding the type of FISH *MYC breakpoints*

	MYC breakpoints (n = *43*)	
	Classical FISH pattern (n = *36*)	*Non-classical FISH pattern* (n = *7*)
Clinical		
> 60 years*	23/36 (72%)	7/7 (100%)
Gender (M:F)	25:11	6:1
Dead of disease	7/20 (35%)	2/5 (40%)
Extranodal location	19/36 (53%)	3/7 (43%)
Immunohistochemical		
CD10+	28/36 (78%)	4/6 (67%)
BCL6+	32/36 (89%)	4/4(100%)
MUM1+	10/30 (3%)	2/4 (50%)
GCB phenotype	28/35 (80%)	4/5 (80%)
BCL2+	28/35 (80%)	6/6 (100%)
MYC+**	21/35 (60%)	7/7 (100%)
Genetics		
IGH/MYC+***	22/30 (73%)	2/6 (33%)
Double/triple hit	23/31 (74%)	4/5 (80%)
MYC genetic heteroclonality	15/36 (42%)	3/7 (43%)

*$P = 0.057$; **$P = 0.040$; ***$P = 0.058$.

double hit or triple hit was associated with a trend toward a worse prognosis compared with other cases carrying *MYC* as a single hit ($P = 0.055$); all patients who died of disease showed a double/triple hit. Double hit and triple hit were found in *MYC HC* (7/14, 50% and 4/14, 29%, respectively) and also in cases without *MYC HC* (12/19, 63% and 1/19, 5%, respectively).

The *classical FISH MYC breakpoint* was found in 36/43 (84%) diffuse large B-cell lymphoma, whereas the *non-classical FISH MYC breakpoint* was detected in 7/43 (16%) cases. Four of the seven cases with *non-classical FISH* pattern showed a break centromeric to the *MYC* breakpoint cluster region, whereas the remaining three had a break telomeric to the *MYC* breakpoint cluster region. Cases with *non-classical FISH MYC breakpoint* were also analyzed using the Abbott *MYC* break-apart probe, confirming the presence of the rearrangement in all cases. No difference was observed in the mean number of *MYC* rearranged cells between cases with *classical* and *non-classical FISH pattern* (74%, range: 40–100%, in *classical FISH MYC breakpoint*, and 81%, range: 46–100%, in *non-classical FISH MYC breakpoint* cases). The comparison of the clinical and pathological characteristics of the diffuse large B-cell lymphoma with both types of *MYC* breakpoints is summarized in Table 3.

In 36 cases, the presence of an *IGH/MYC* dual fusion was studied. Twenty-four cases (67%) presented the specific translocation t(8;14), whereas 12 cases had non-*IGH* rearrangements. The *IGH/MYC* translocation was observed in the majority of cases with a *classical FISH MYC breakpoint* (22/30, 73%), but only in 2/6 (33%) cases with a *non-classical FISH MYC breakpoint* ($P = 0.058$, Table 3). An *IGH/MYC* translocation was observed in 18/22 (82%) cases without *MYC HC,* but in 6/14 (43%) cases with *MYC HC* ($P = 0.016$, Table 2). Sixteen of 22 (73%) cases with *IGH/MYC* translocation and 6/8 (75%) cases without an *IGH/MYC* showed double/triple hit. The presence of an *IGH/MYC* translocation did not significantly correlate with overall survival or other clinical–pathological features (Supplementary Table 3).

Correlation Between MYC Expression and *MYC* Genetic Status in Diffuse Large B-Cell Lymphoma

Nine of 14 (64%) cases with lower MYC expression (< 40% of stained cells, classified as MYC immunohistochemistry negative) had *MYC HC* ($P = 0.026$) (Table 1) and the *MYC founder* clone was in the range of 7–60% (mean: 22%, median: 18%). Five cases with low expression and without *MYC HC* showed the *MYC founder* clone in the range of 46–91% (mean: 73% and median: 74%).

In contrast, only 8/28 (29%) cases with positive MYC expression had *MYC HC*. In these cases, the *MYC founder* clone was in the range of 5–33% of cells (mean: 21% and median: 23%). The remaining cases with positive expression but no *MYC HC* showed the *MYC founder* clone in the range of 40–98% of cells (mean and median: 71%). FISH and immunohistochemistry images of two cases with *MYC HC* are shown in Figures 2a–d.

Owing to the high chromosomal complexity involving *MYC* in *heteroclonal* cases, it was not possible to establish specific correlations between other single clones or a specific combination of these clones and MYC protein expression. Notably, the FISH pattern with loss of red signal (corresponding to the derivative chromosome carrying *MYC* telomeric portion) characterized 5/14 (36%) cases with lower MYC expression ($P = 0.003$).

Considering the breakpoint type observed by FISH, MYC protein staining was positive in all cases (7/7) with a *non-classical FISH MYC breakpoint* (MYC expression in the range of 42–97% tumor cells; mean: 85%, median: 93%), and in 21/35 (60%) cases with the *classical breakpoint* (MYC expression in the range of 43–98%; mean: 74%, median: 79%). The 14 cases classified as negative for MYC expression had all the *classical FISH MYC breakpoint* ($P = 0.040$).

No difference was observed in MYC protein expression according to *IGH* or *non-IGH* genes as the *MYC* translocation partner. Sixteen of 25 (64%) cases with positive MYC expression and 8/11 (73%) cases with negative expression showed *IGH/MYC* translocation. Cases with *IGH/MYC* translocation had a similar number of MYC stained cells (43–97%, mean: 73%, median: 75%)

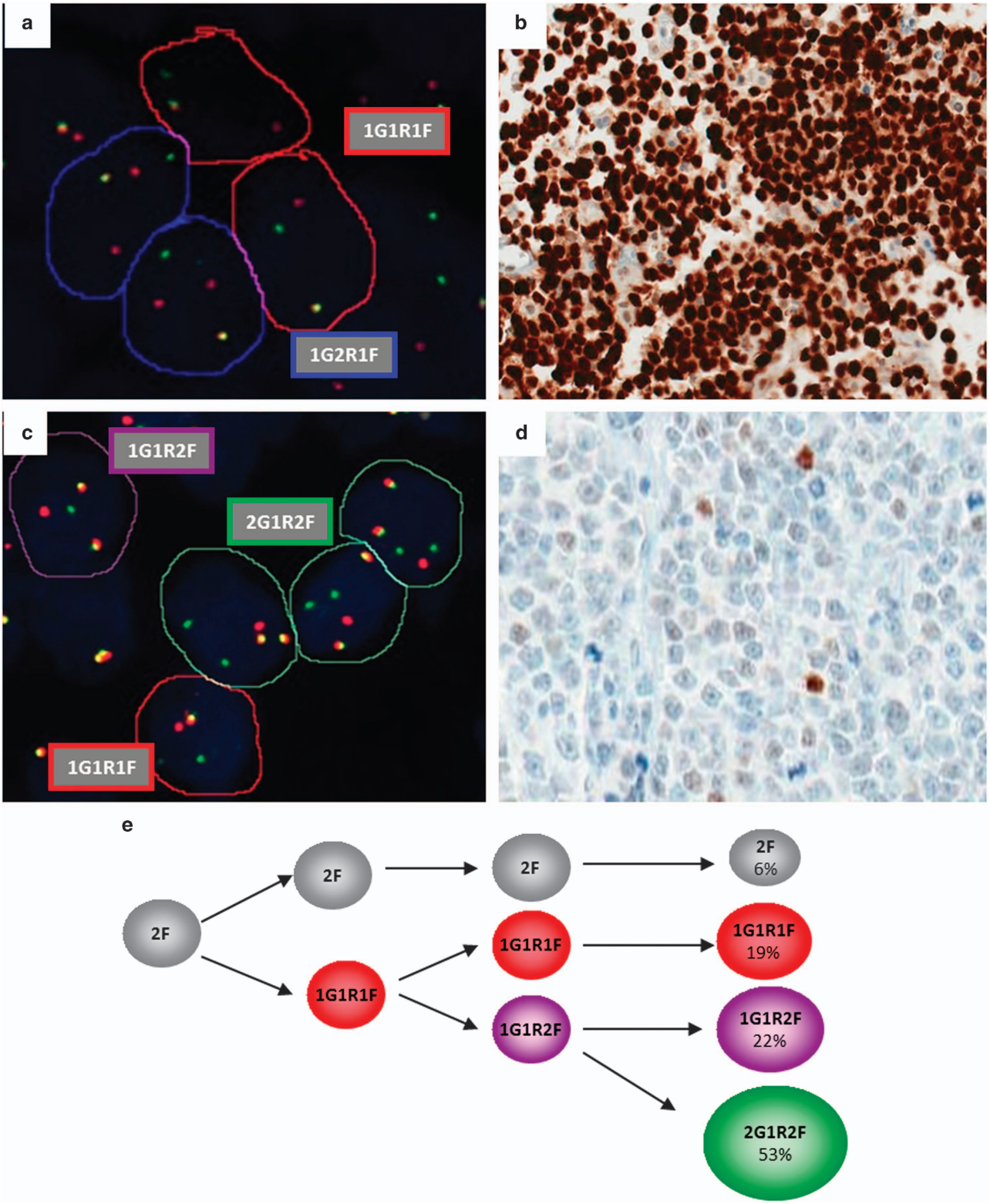

Figure 2 FISH and immunohistochemistry images of two diffuse large B-cell lymphoma captured by digital system (Bioview and Aperio, respectively); (**a**, **b**) case with *MYC genetic heteroclonality* and positive MYC expression; (**c**, **d**) case with *MYC genetic heteroclonality* and negative (low) MYC expression; (**e**) schematic example for tumoral growth and clonal evolution leading to FISH pattern observed in (**c**) (case #9 of Table 1).

than those with non-*IG* partner (42–98%, mean: 84%, median: 93%).

Clinical, Pathological and Genetic Characteristics of Burkitt Lymphoma

The control group comprised 14 Burkitt lymphoma patients, 10 males and 4 females, with a mean age of 41 years (range: 7–83). Extranodal presentation was observed in 10/13 (77%) patients. All samples were positive for CD20 and CD10. BCL6 was also positive in 12/13 (92%) cases and MUM1 expression was present in 6/11(55%) cases. The proliferation index (Ki-67) was higher than 90% in 12/13 (92%) cases, and EBV infection was detected in 3/12 (25%) cases. MYC expression was evaluable in 11 of 14 cases, and all cases were positive, showing a range of 57–95% stained cells (mean: 83%, median: 87%). *MYC* rearrangement by FISH analysis was found in a range of 65–95% tumor cells (mean: of 83%, median: 85%). *MYC HC* was not observed in any Burkitt lymphoma case. All Burkitt lymphoma were characterized by a uniform population carrying the *MYC* rearrangement without additional alterations (all raw data available in Supplementary Table 2).

Ten of 14 (71%) Burkitt lymphoma cases showed a *classical FISH MYC breakpoint* and 4 of 14 (29%) a *non-classical FISH MYC breakpoint*. Among the latter cases, two showed a break in the region centromeric to MYC breakpoint cluster region, and two in the telomeric one (similarly to diffuse large B-cell lymphoma, these cases were confirmed using the *MYC* break-apart probe from Abbott Molecular). The cases with a *classical FISH MYC breakpoint* showed the rearrangement in 65–95% of cells (mean: 87%, median: 91%), and those with a *non-classical FISH MYC breakpoint* in 67–79% of the cells (mean and median: 72%). Cases with the *classical FISH MYC breakpoint* had MYC expression in 57–95% of cells (mean: 87%, median: 92%), and in those with a *non-classical FISH MYC breakpoint*, protein expression was observed in 60–84% of cells (mean: 73%, median: 75%). An *IGH/MYC* dual fusion probe was used in 10 cases, and 8 (80%) demonstrated the specific translocation t(8;14)(q24;q32). Seven of eight (88%) cases with *IGH/MYC* carried the *classical FISH MYC breakpoint*. No clinical or pathological differences according to type of *MYC* breakpoint was observed in Burkitt lymphoma cases.

Discussion

Diffuse large B-cell lymphoma represents an exceedingly heterogeneous disease both at the clinical and biological levels. Despite the corroborating role of MYC alterations as a prognostic marker for diffuse large B-cell lymphoma,[1–5] its assessment represents a major challenge in clinical practice. A novel commercial monoclonal antibody has improved the detection of MYC protein expression,[6–7] however, immunohistochemistry assessment and quantification still remains difficult because of highly variable MYC expression.[11–12] In this regard, image digital analysis and computerized assisted interpretation may prove to be a valid aid. Applying this strategy (Aperio) to our series of 43 *MYC* rearranged diffuse large B-cell lymphoma, we observed high (>40%, immunohistochemistry positive) and low (<40%, immunohistochemistry negative) MYC protein expression in 88% and 12% cases, respectively, confirming previous observations.[5,8–10] Interestingly, even if all of the investigated diffuse large B-cell lymphoma samples had a *MYC* translocation, great differences were observed in terms of the frequency of MYC expressing cells: in MYC immunohistochemistry positive cases, the median number of stained cells was 86%, whereas it was 17% in MYC immunohistochemistry negative cases. To better understand the possible role of the *MYC* gene in the variability of MYC protein expression, we performed a detailed investigation of *MYC* rearrangement in the whole tumoral context, as well as in single-cell details.

Relative to *MYC* rearrangement, regardless of the specific type of FISH pattern, the percentage of rearranged cells in our diffuse large B-cell lymphoma cases ranged from 40% to 98%. Using a computerized imaging system (Bioview), we were able to specifically describe and quantify all the different populations of cells within the same tumor, distinguishing the *MYC founder* clone (*MYC* rearrangement alone) from other populations of cells with *MYC* complex patterns, showing the *MYC* translocation coupled to further alterations involving *MYC*.

Reproducing the hypothesis of a clonal evolution model with a founding clone and multiple subclones, as described for other hematologic malignancies,[32,33] we hypothesize that during tumor growth, the first pathogenetic event (*MYC* translocation) is followed by subsequent changes involving the *MYC* derivatives or the normal allele (gain/loss or extra copies, respectively). The expansion of these descending cells results in new subclones similar to the progenitor (sharing *MYC* translocation) but also different (carrying additional modifications) (Figure 2e). This picture defines the presence of *MYC genetic heteroclonality/HC*, distinguishing this precise status from the more general concept of genetic heterogeneity. Results from conventional cytogenetic studies (http://cgap.nci.nih.gov/Chromosomes/CytList) reinforce this hypothesis, and our findings confirm that FISH is a valid approach to characterize the presence of *MYC HC* in tissue samples.

MYC HC was observed in 42% of diffuse large B-cell lymphoma and correlated with lower MYC expression. In contrast, the vast majority of cases with MYC expression (80%) did not show *MYC HC*. In cases with *MYC HC*, the *MYC founder* clone represented a minor population, corresponding to a median of 20% of tumor cells. Interestingly, in the

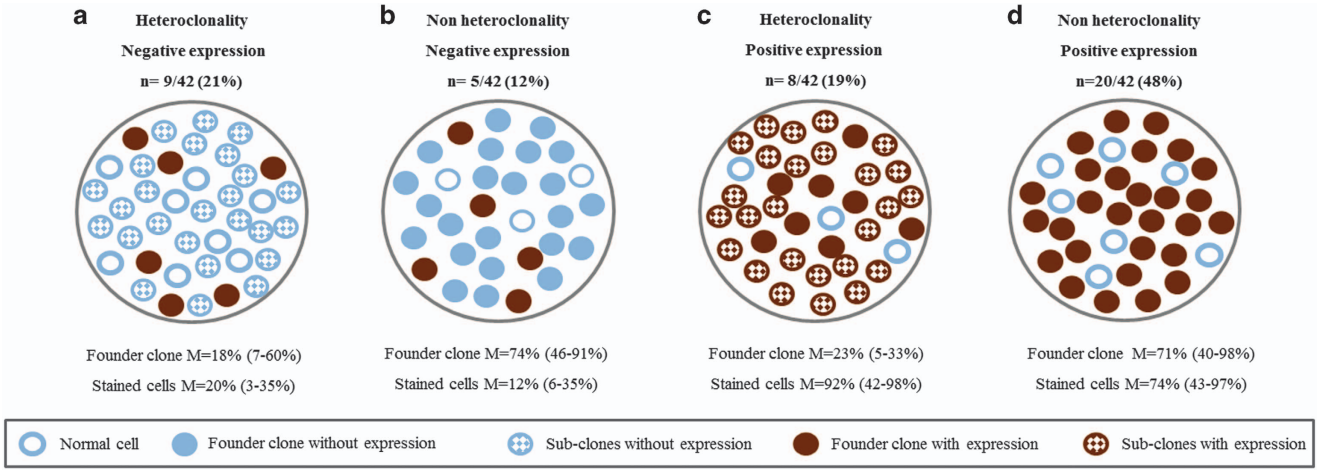

Figure 3 Putative model to describe the impact of the presence of *MYC genetic heteroclonality* on MYC protein expression in diffuse large B-cell lymphoma based on the percentage of rearranged and stained cells. M, median.

heteroclonal cases with lower expression, the dimensions of the founder clone and of the MYC stained population nearly overlapped (median 18% and 20%, respectively) (Figure 3a). Similarly, in cases without *MYC HC* and positive for MYC expression, the dimension of the *MYC founder* clone and the number of overexpressing cells were very similar (71% *vs* 74%) (Figure 3d). In Burkitt lymphoma, this phenomenon was even more evident, that is, all cases lacked *heteroclonality*, had positive expression and showed the *MYC founder* clone as a major population (85% and 87%, respectively). In other words, this precise quantification and comparison revealed that in about two-thirds of diffuse large B-cell lymphoma cases with *MYC* rearrangement, the relationship between founder and expressing clones was strong: the smaller or bigger the first, the smaller or bigger the second. In contrast, in the subgroup of cases without *MYC HC* and lower MYC expression (corresponding to about 10% of diffuse large B-cell lymphoma), the dimension of the *MYC founder* clone was completely different from the expressing population, with the former exceeding by six times the latter (median dimension: 74% *vs* 12%) (Figure 3b); the opposite was observed in the subgroup of cases with *MYC HC* but positive MYC expression (about 20%) wherein the number of expressing cells highly outnumbered that of the founder clone (median 23% vs 92%, respectively) (Figure 3c). All these data suggest that MYC protein expression may be affected by the presence of *heteroclonality* (see a putative model in Figure 3).

From a biological point of view, changes in the synthesis of MYC protein may be related to primary events affecting the founder clone itself, or to secondary events occurring in the derived subclones during tumor growth. In fact, the majority of *MYC HC* showed a reduced expression, indicating the occurrence in subclone of modifications at the post-transcriptional/translational level or strictly affecting *MYC* gene itself. The fact that five cases with low MYC expression were characterized by loss of the red derivative (which is the one that contains the coding portion of *MYC* juxtaposed to the promoter of the partner gene) may support this hypothesis. In contrast, in those *HC* cases still expressing MYC protein, the subclones are likely not influenced by changes and remain capable of maintaining the expression. Although the mechanisms responsible for the loss of MYC expression are still unclear, the presence of *heteroclonality* may explain the low MYC expression in 10–30% of diffuse large B-cell lymphoma with *MYC* rearrangement.[5,8–12,26] Further studies using combined method of FISH and immunofluorescence (such as FICTION technique) may help to characterize more precisely the relationship between MYC gene and protein status in all these different clones.

MYC HC was observed with similar frequencies in cases showing *MYC* rearrangement with *classical* or *non-classical FISH breakpoints* (42% and 43% of cases, respectively), thus indicating that the breakpoint does not have a role in clonal evolution. Interestingly, *MYC HC* cases were primarily partnered with a non-*IGH* rearrangement, whereas the most recurrent partner of cases without *MYC HC* was the *IGH* gene, similar to Burkitt lymphoma in our series, suggesting that *IGH-MYC* fusion represents a crucial driver that remains stable during tumoral expansion without evolving in subclones. Overall, the *IGH* partner does not seem to influence protein expression in our series of diffuse large B-cell lymphoma, but in cases without *HC* it was associated with higher MYC expression, corroborating findings of a recent study.[26]

Despite the fact that no peculiar clinical–pathological features have been observed in diffuse large B-cell lymphoma with *MYC HC*, all the aforementioned characteristics may not only reflect the

complex biology of this disease, but also may provide a useful tool for defining diffuse large B-cell lymphoma subgroups. In particular, the recognition of *MYC HC* may be a diagnostic clue for daily practice as it is absent in Burkitt lymphoma.

Further to *genetic heteroclonality*, the FISH approach we used to investigate the *MYC* genetic status at the single-cell level allowed the determination of the presence of different breakpoints that may affect *MYC* rearrangement. In this context, it is important to underline that FISH is an indirect method for breakpoint description and is probe related; therefore, the terms '*classical* vs *non-classical FISH MYC breakpoint*' that we defined in this study should be intended only referring to *MYC* split signal probe that we used.

In our cohort, we observed that the most common breakpoints occur in, or close to, the *MYC* breakpoint cluster region (*classical* FISH *MYC breakpoint*) both in diffuse large B-cell lymphoma (84%) and in Burkitt lymphoma (71%), and in these cases the preferential translocation partner was the *IGH* gene. Non-classical disruptions seems to affect a significant subgroup of patients (16% of diffuse large B-cell lymphoma and 29% of Burkitt lymphoma), especially those diffuse large B-cell lymphoma with *MYC* rearrangements involving a *non-IGH* gene ($P = 0.058$), confirming literature data.[15,24–25,34] Of note, in our cohort all 14 diffuse large B-cell lymphoma with *non-classical FISH breakpoint* had a positive MYC expression, whereas all 7 cases with low expression had a *classical* break ($P = 0.040$), thus indicating that a *non-classical* break does not influence protein expression and again confirming that loss of MYC expression may be related to more complex post-transcriptional/translational mechanisms. No correlation was observed between clinical–pathological characteristics and different types of breakpoints, but *non-classical FISH breakpoints* seemed more frequent in diffuse large B-cell lymphoma patients older than 60 years; larger studies are needed to assess the possible biological reasons for this observation.

Finally, we investigated the potential relationship between *MYC* rearrangement and the concomitant presence of *BCL2* and/or *BCL6* rearrangements by virtue of their common negative prognostic value for diffuse large B-cell lymphoma. In accordance with the literature, double hit and triple hit were frequently observed in our cohort (58% and 15%, respectively) and indicated a poor prognosis,[35] but the presence of double hit or triple hit was neither correlated with *MYC HC* nor with the type of FISH *MYC* breakpoints or partners, confirming the biological complexity of this disease.

In conclusion, *MYC* status reflects complex genomic alteration mechanisms in diffuse large B-cell lymphoma that may specifically rebound on *MYC* rearrangement itself (such as *non-classical breakpoints* and/or different partners) or globally to its distribution in tumoral cell populations. Our study defines, for the first time, the presence of *MYC genetic heteroclonality* in a large series of diffuse large B-cell lymphoma positive for *MYC* rearrangement. *MYC HC* is a frequent event in diffuse large B-cell lymphoma and has a relevant role in modulating MYC protein expression. Prospective studies of larger cohorts are needed to corroborate these findings and to understand their potential clinical relevance.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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